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Stable Human Immunodeficiency Virus Type 1 (HIV-1) Resistance in Transformed CD4$^+$ Monocytic Cells Treated with Multitargeting HIV-1 Antisense Sequences Incorporated into U1 snRNA

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We have approached the development of a human immunodeficiency virus type 1 (HIV-1) therapeutic product by producing immune cells stably resistant to HIV-1. Promonocytic CD4$^+$ cells (U937) were made resistant to HIV-1 by the introduction of a DNA construct (pNDU1A,B,C) that contained three independent antisense sequences directed against two functional regions, transactivation response and *tat/rev*, of the HIV-1 target. Each sequence was incorporated into the transcribed region of a U1 snRNA gene to generate U1/HIV antisense RNA. Stably transfected cells expressed all three U1/HIV antisense transcripts, and these transcripts accumulated in the nucleus. These cells were subjected to two successive challenges with HIV-1 (BAL strain). The surviving cells showed normal growth characteristics and have retained their CD4$^+$ phenotype. In situ hybridization assays showed that essentially all of the surviving cells produced U1/HIV antisense RNA. No detectable p24 antigen was observed, no syncytium formation was observed, and PCR-amplified HIV *gag* sequences were not detected. Rechallenge with HIV-1 (IIIB strain) similarly yielded no infection at a relatively high multiplicity of infection. As a further demonstration that the antisense RNA directed against HIV-1 was functioning in these transfected immune cells, Tat-activated expression of chloramphenicol acetyltransferase was shown to be specifically inhibited in cells expressing Tat and transactivation response region antisense sequences.

The incidence of human immunodeficiency virus (HIV) infection has reached alarmingly high levels worldwide, prompting efforts to develop effective treatments. Much of the development has focused on an antiviral drug-based approach designed for the most part to slow viral growth. We have approached the development of an effective therapeutic product by focusing on rendering the immune cells refractory to HIV type 1 (HIV-1) infection. This will permit reconstitution of a population of immune cells in the presence of HIV-1 and may act to restore immunocompetence. This treatment can be implemented by ex vivo treatment of hematopoietic stem cells to add specific genetic material (4, 13).

As a means of inducing resistance to HIV-1, monocytic cells in culture were transfected with a DNA construct expressing three different sequences, each directed against a critical HIV-1 target sequence. This multitargeting approach was used in order to combat the high rate of variability and mutability of HIV-1 that often results in resistance to therapies against a single virus target (6, 19). The anti-HIV-1 sequences were incorporated into U1 snRNA, a stable and abundant snRNA molecule that functions in RNA processing (3, 23), to generate U1/HIV antisense RNA. U1 snRNA transcripts are synthesized in the nucleus and transported to the cytoplasm, where they undergo modifications; after binding of specific proteins, they are reimported to the nucleus, where they function with other snRNPs in RNA splicing (23). Due to the central role in RNA processing played by U1 snRNA, the U1 promoter is expressed in all cell types. Approximately 10^6 copies of U1 RNA are transcribed from approximately 30 U1 genes per cell. Each promoter thus transcribes an average of approximately 3×10^4 copies per generation (3).

We report here the results obtained by using independent multitargeting U1/HIV antisense constructs to confer stable HIV-1 resistance to CD4$^+$ cells.

MATERIALS AND METHODS

Construction of plasmids with antisense sequences introduced into the transcript region of the U1 gene. The steps used are presented in Fig. 1. The U1 gene used is derived from pHSD-4 (10). Four pairs of oligonucleotides, designated HVA-1 and HVA-2, HVB-1 and HVB-2, HVC-1 and HVC-2, and HVD-1 and HVD-2, were synthesized. Each pair was hybridized to form double-stranded molecules containing termini compatible with the *Bcl*I and *Bsp*E1 ends produced from restriction enzyme digestion of the U1 transcript region of the plasmid. Sequence A was taken from the HIV-1 transactivation response region of the HXB2 strain, a T-cell-tropic clone of isolate IIIB (2), sequence B was taken from the tat/rev exon of the LA1 strain, also a T-cell-tropic isolate (5), and sequence C was taken from the tat/rev splice acceptor sequence of the BAL strain, a monocytotropic isolate. Sequence D contains sequences unrelated to either U1 or HIV and serves as a negative control. Sequences of the oligonucleotides are as follows: HVA-1, 5′ GAT CCG GAT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT 3′; HVA-2, 5′ CCG GAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAG TGC TTA AGC CTC AAT CCG 3′; HVB-1, GAT CCG GAC CTT GAG GAG GTC TTC GTC GCT GTC TCC GCT TCT TCC TGC CAT AGG AGA GCC TAA GGT 3′; HVB-2, 5′ CCG GAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA GGT CCG 3′; HVC-1, 5′ GAT CCG GAT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT 3′; HVC-2, CCG GAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC ATC CG 3′; HVD-1, 5′ GAT CAG CAT GCC TGC AGG TCG ACT CTA GAC CCG GGT ACC GAG CTC GCC CTA TAG TGA GTC GTA TTA T 3′, and HVD-2, 5′ CCG GAT AAT ACG ACT CAC TAT AGG GCG AGC TCG GTA CCC GGG TCT AGA GTC GAC CTG CAG GCA TGC T 3′.

*Bcl*I and *Bsp*E1 restriction enzyme digestion of pUC13 carrying the U1 operon removes a 41-bp segment (bases 31 through 71) from the transcribed region of

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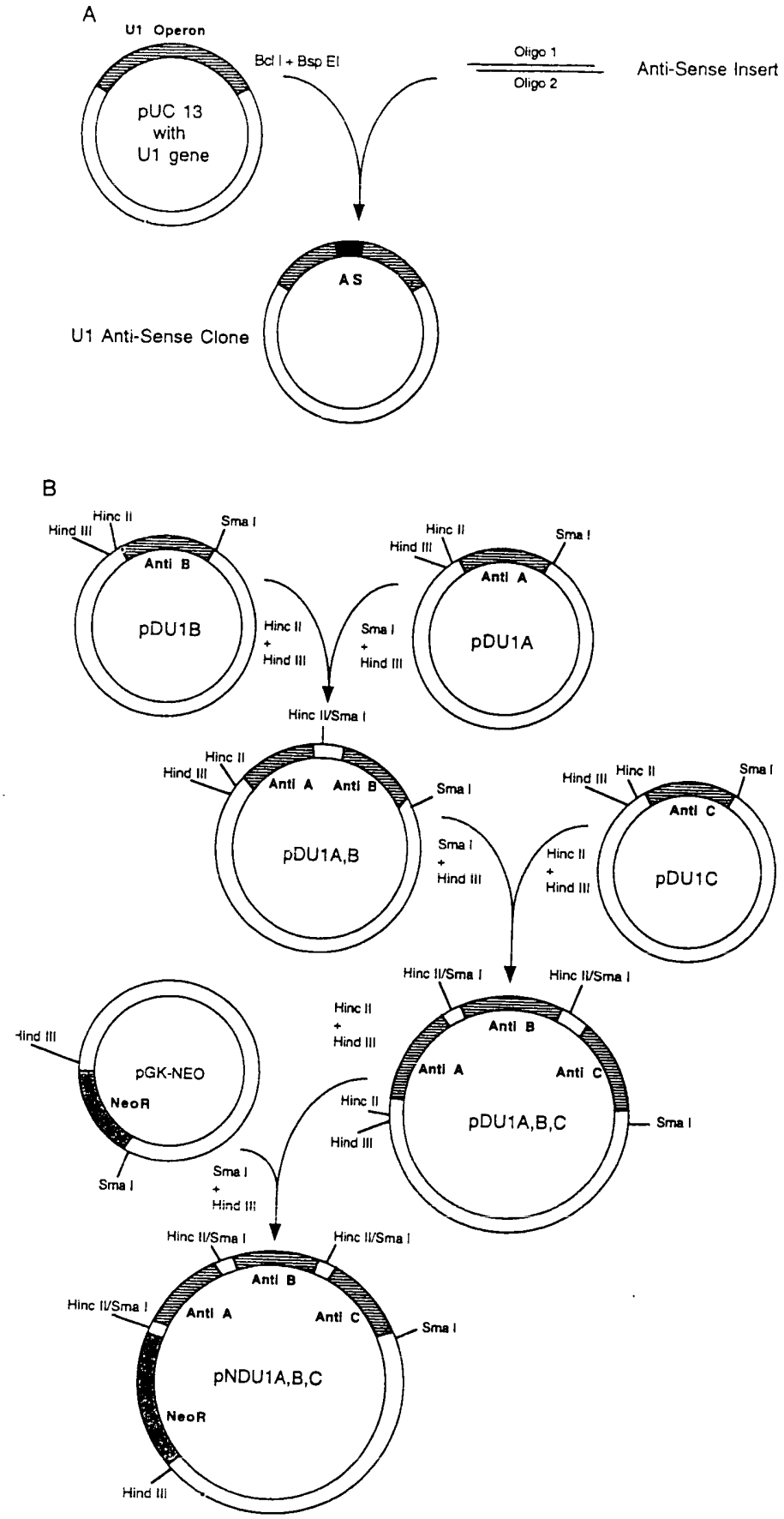
A
U1 Operon
pUC 13
with
U1 gene
Bcl I + Bsp EI
Oligo 1
Oligo 2
Anti-Sense Insert
AS
U1 Anti-Sense Clone
B
Hinc II
Hind III
Sma I
Anti B
pDU1B
Hinc II
+
Hind III
Sma I
+
Hind III
Anti A
pDU1A
Hinc II/Sma I
Anti A
Anti B
pDU1A,B
Anti C
pDU1C
pGK-NEO
NeoR
Anti A
Anti B
Anti C
pDU1A,B,C
pNDU1A,B,C

the U1 gene. Each of the hybridized oligomer pairs was inserted into the *BclI*/*Bsp*E1 site created in the U1 transcript region of pHSD-4 to yield plasmids designated pDU1A, pDU1B, pDU1C, and pDU1D. A segment carrying the neomycin resistance gene was derived from pGK-neo (10) and introduced into each plasmid (Fig. 1). The resulting plasmids were designated pNDU1A, pNDU1B, pNDU1C, and pNDU1D. The three U1/HIV antisense cassettes derived from pDU1A, pDU1B, and pDU1C were assembled into a single construct (pNDU1A,B,C) as shown in Fig. 1.

U937 cells carrying U1/HIV antisense constructs. U937 cells (9) were transfected with pNDU1A,B,C by the Lipofectin procedure (Bethesda Research Laboratories [BRL]). The treated cells (5×10^5) were inoculated into T-25 flasks (Corning) containing culture medium (Dulbecco modified Eagle medium; Gibco and BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco and BRL) and 600 μg of G418 (Gibco and BRL) per ml. The G418-containing medium was replaced every 3 to 4 days. The resulting population of G418-resistant U937 cells was designated 2.10.16. Selection of a population of cells carrying pNDU1D (designated 2.2.78) was done by using Lipofectin and enrichment for G418 resistance as described above for 2.10.16.

PCR in situ hybridization for HIV-1 DNA sequences. PCR in situ hybridization assays for U1/HIV antisense RNA were performed as described by Nuovo et al. (14, 16). Cells obtained from the late stage of the growth phase were fixed for 15 to 24 h in 10% buffered formalin and then washed twice in diethyl pyrocarbonate-containing water. Approximately 5,000 cells were placed on a silane-coated microscope slide and digested in 2 mg of pepsin per ml at 37°C for 30 min. Assays for HIV-1 DNA were performed by using hot-start PCR (35 cycles) employing the gag primers SK38 and SK39 or SK145 and SK431 (nucleotides 1369 to 1395 and 1507 to 1481). PCR in situ hybridization for the provirus was done with digoxigenin-labeled probes (SK19 and SK102) to assay for viral entry after challenge.

In situ RT-PCR assay for U1/HIV antisense RNA sequence. The procedure described above for in situ PCR detection of DNA was modified for specific detection of U1/HIV antisense RNA sequences. Cells fixed to silane-coated glass slides were protease treated and incubated overnight in RNase-free DNase solution (10 U/section; Boehringer Mannheim) at 37°C to eliminate nonspecific DNA repair and mispriming. This allowed for target-specific direct incorporation of digoxigenin-dUTP (used at a concentration of 10 μM in the reverse transcription [RT]-PCR mix). Primers specific for U1/HIV antisense RNA sequences were used. Assays for U1/HIV-1 RNA sequences used primer HU1, which comprises a sequence from the transcript region of U1 (5′-CCTGGCAGGGG AGATACCATG-3′), as the upstream primer and either HVA-2, HVB-2, or HVC-2 as the downstream primer. For cells transfected with HVA, the HVB-2 and HVC-2 primers served as negative controls; similarly, for HVB transfectants, HVA-2 and HVC-2 served as controls, and for HVC transfectants, HVA-2 and HVB-2 served as negative controls. After protease and DNase digestion, RT and PCR were accomplished by using an rTth EZ kit from Perkin-Elmer as previously reported (15). Pooled normal lymphocytes infected with HIV-1 isolate IIIB for 4 days, and sham-infected cells (kindly provided by Roy Steigbigel, State University of New York at Stony Brook) served as additional controls. The negative and positive controls consistently yielded the expected results. The signal is manifested as a blue precipitate due to the action of the antidigoxigenin-alkaline phosphatase conjugate on the chromogen, nitroblue tetrazolium, and the substrate, β-chloroindolylphosphate. Negative cells stained pink due to the counterstain, nuclear fast red.

Assay for HIV-1 DNA. PCR was performed as described by Schnittman et al. (18), using primer pairs SK38 and SK39 (*gag*). Reaction products were analyzed by electrophoresis in 1.5% agarose in the presence of ethidium bromide. Comparisons were made with amplified DNA from U1.1A, a chronically infected promonocyte cell line which contains two integrated HIV copies per cell (8). U1.1A cells and U937 cells were mixed in various proportions so that in all assays, DNA from 10^5 cells was analyzed.

Transient expression assays for the effect of U1/HIV antisense RNA on Tat-activated expression of CAT. (i) Plasmids. The HIV-1 long terminal repeat-chloramphenicol acetyltransferase (CAT) reporter construct pU3III-CAT was a gift from Craig Rosen (17). The Tat plasmid pCV-1 contains a 1.8-kb fragment of HIV-1 cDNA encompassing the tat gene (1).

(ii) DEAE-dextran transfection and CAT assay. In each transfection, 2.5×10^6 cells per condition were used. The cells were washed with serum-free RPMI 1640 (sfRPMI) and resuspended in 1 ml of sfRPMI containing 400 μg of DEAE-dextran (Sigma) in 50 mM Tris-HCl (pH 7.3) with 2.5 μg of total plasmid per condition. Cells were then incubated at 37°C for 1 h, washed with sfRPMI, resuspended in fresh culture medium, and incubated at 37°C for 40 to 45 h. The cells were harvested, washed, and resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8), and cell extracts were prepared by three cycles of freezing (dry ice-ethanol) and thawing (37°C water bath). Extracts were centrifuged, and the protein content in the supernatants was estimated by using a micro bicinchoninic acid reagent kit (Pierce, Rockford, Ill.).

(iii) CAT activity assay. CAT activity was determined by incubating 25 μg of cell extracts with [^{14}C]chloramphenicol (57.9 mCi/mmol; NEN) and 2.5 mM acetyl coenzyme A (Pharmacia) at 37°C for 2 h. Incubation was followed by extraction with ethyl acetate and ascending thin-layer chromatography. Chromatograms were autoradiographed, and areas of radioactivity were cut out and counted in scintillation fluid for quantification of CAT expression.

HIV-1 challenge assays. Cells (0.5×10^6 cells) were incubated with the BAL or IIIB strain of HIV-1 at various multiplicities of infection (MOI, calculated with perpheral mononuclear cells) in the presence of 2 μg of Polybrene per ml for 2 h at 37°C, using the procedure of Laurence et al. (9). The cells were then washed and resuspended in 1 ml of culture medium (RPMI 1640 containing 10% fetal bovine serum [Flow Laboratories] for U937 and the same components plus G418 for all transfected cells). The cells were plated in duplicate (0.5 ml per well). One half of the culture medium was removed and replaced with fresh medium every 3 or 4 days. At intervals postinfection, samples of these cells were tested for virus growth by assaying for p24 by enzyme-linked immunosorbent antigen capture as instructed by the manufacturer (DuPont). Cells were also tested for viability by using trypan blue dye exclusion. The levels of G418 used had no effect on susceptibility of cells to infection or level of p24 antigen produced.

RESULTS

Preparation of a multitargeting U1/HIV antisense construct. HIV-1 antisense sequences were inserted into the U1 transcript sequence as depicted in Fig. 1A. The 41 bp removed from the transcript region were replaced with the antisense oligonucleotides sequences A (68 bp), B (62 bp), C (61 bp), and D (63 bp). These insertions result in an increase of 27 bases in the length of the U1 transcript region of pDU1A, 21 bases in pDU1B, 20 bases in pDU1C, and 22 bases in pDU1D. The effect of these replacements on U1 RNA structure was assessed by the McDNASIS program (Hitachi, Inc.). The predicted secondary structures in all cases indicated that the insertions do not alter the secondary structure of the 3′ end; i.e., the U1/HIV antisense molecules are predicted to form normal stem-loops III and IV as well as a normal Sm region.

In situ assays for U1/HIV antisense RNA in 2.10.16 cells transfected with pNDU1A,B,C. To mimic more closely the expected therapeutic use of these constructs, a pool of transformed clones (designated 2.10.16) was obtained by transfection of U937 cells with plasmid pNDU1A,B,C and subsequent enrichment of the transfected population for G418 resistance (Materials and Methods). Figure 2 shows expression of the U1/HIV antisense sequences in 2.10.16 as analyzed by in situ amplification assay. These experiments used primers HU1-1 and HVA-2. Similar results were obtained when either HVB-2 or HVC-2 was used as the downstream primer. Note the nuclear localization of the dark blue precipitate indicative of the PCR-amplified cDNA (Fig. 2D). Nuclear staining (hematoxylin and eosin) indicates the large area of the cells occupied by the nucleus (Fig. 2A). It is evident that 10 to 20% of the 2.10.16 cells express transcripts corresponding to the U1/HIV inserts at a high enough level to be detected by this assay. 2.2.78 cells (containing the control plasmid) showed no signal (Fig. 2C), and omission of reverse transcriptase from the amplification procedure resulted in no signal in 2.10.16 cells (Fig. 2E).

HIV challenge of 2.10.16 cells. The 2.10.16 cells were then exposed to the BAL strain of HIV-1 at an MOI of 0.15. As can be seen in Table 1 (challenge 1a), this pooled clone 2.10.16 showed resistance to HIV-1, as indicated by a reduction in p24 production compared to the control clones (either U937 or

FIG. 1. Insertion of antisense (AS) sequences into U1 operons. (A) Hybridization of the deoxyoligonucleotide (Oligo) pairs resulted in double-stranded molecules with overhanging single-stranded termini compatible with the *Bcl*I and *Bsp*E1 ends produced from the restriction enzyme digestion of the U1 transcript region of the plasmid. The resulting plasmids containing the inserts were designated pDU1A, pDU1B, pDU1C, and pDU1D. (B) Assembly of a DNA construct (pNDU1A,B,C) containing three U1/HIV antisense cassettes.

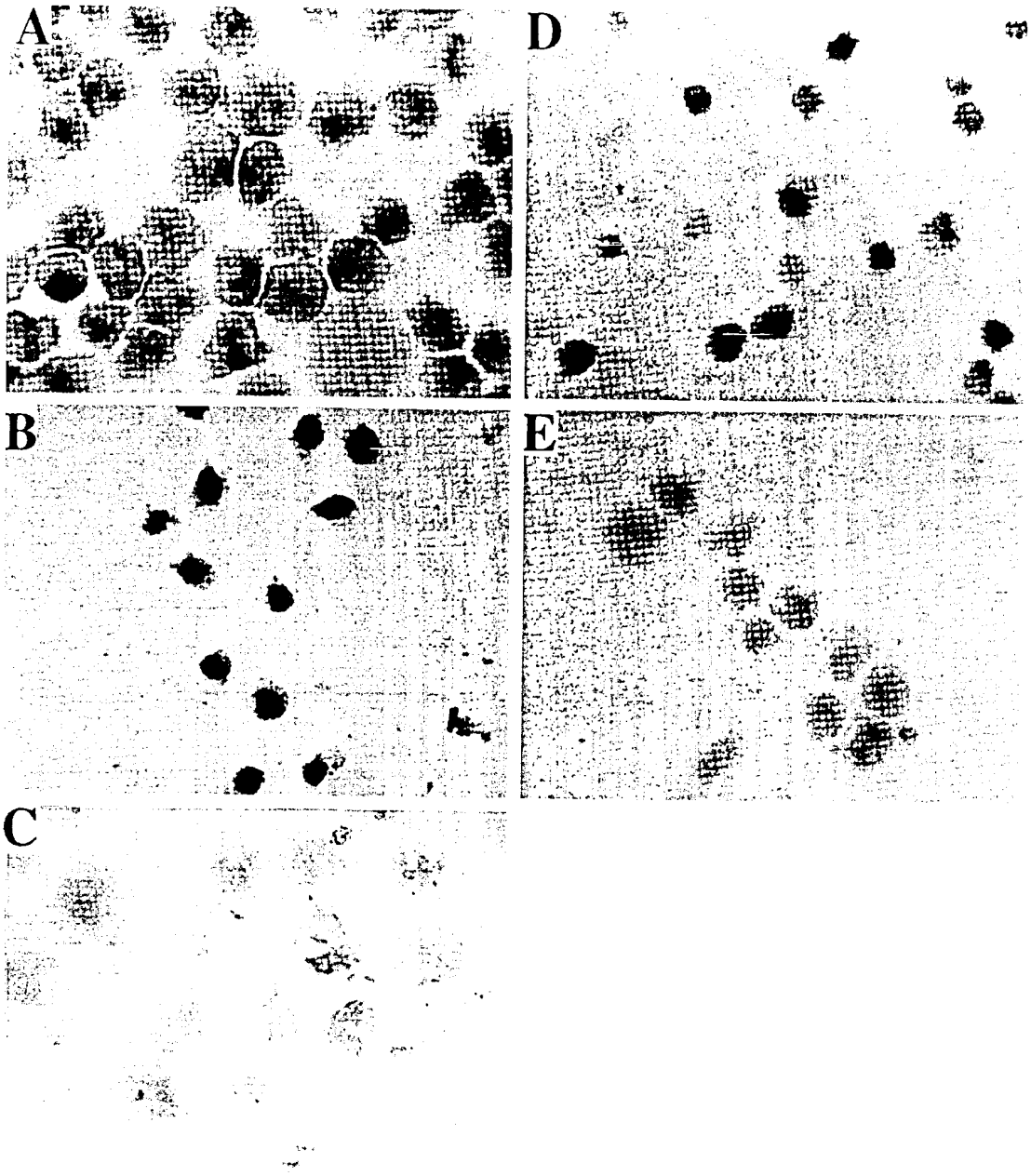

FIG. 2. In situ RT-PCR assay for U1/HIV antisense RNA in 2.10.16 (unchallenged) and 2.10.16R2 (challenged) cells. (A) Nuclear staining shows the cytologic features of the 2.10.16 cells after staining with hematoxylin and eosin. Note the large blue nucleus and the thin rim of pink cytoplasm. (B) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16R2 (postchallenge) cells revealing a strong nuclear signal in all cells. (C) In situ RT-PCR for U1/HIV antisense sequence A in 2.2.78 cells. (D) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16 (prechallenge) cells. Note that a minority of the cells have a detectable nuclear signal. (E) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16 cells when the reverse transcriptase treatment step is omitted.

2.2.78) at 6 days postinfection. In another challenge experiment (Table 1, challenge 1b), 2.10.16 cells showed greater than 90% inhibition of p24 production in the culture (compared with parent U937 cells and 2.2.78 cells) at 16 days postinfection. Cell viability assays performed on day 24 postinfection by using trypan blue exclusion showed that 17% of 2.2.78 pool controls from challenge 1b were viable. The pooled clone 2.10.16 were 40 to 60% viable and had no visible syncytia (data not shown).

At day 24, the 2.2.78 pool (which contains a DNA sequence unrelated to either HIV-1 or U1 inserted into the U1 transcript region [see Materials and Methods]) and the 2.10.16 cells were subjected to Ficoll gradient separation to separate live cells from dead cells, and the procedure was continued as a routine maintenance procedure as necessary until day 35. At day 35, there were no live cells remaining in the 2.2.78 culture.

Second challenge of 2.10.16 and U937 control cells. The pooled clone population 2.10.16 taken from day 31 postinfection (now termed clone 2.10.16R1) and U937 control cells (2.2.78) were subjected to a second challenge with the HIV-1 BAL strain at an MOI of 0.1. The cells were infected and maintained as before. Results of p24 assays at days 9 and 12

TABLE 1. HIV challenge of U937, 2.10.16, and 2.2.78 cells

Cells	p24 (pg/ml)		% Viable cells,[a] challenge 1b, day 24
	Challenge 1a, day 6	Challenge 1b, day 16	
U937	959	200	0
2.10.16 pooled clones	514 (46.6[b])	12 (94.5)	40–60
	554 (42.2)		
2.2.78 pool control	780	220	17

[a] Assayed by trypan blue staining assay.
[b] Percent difference in amount of p24/ml when the 2.10.16R1 cell supernatant is compared with the supernatant from U937 cells.

postinfection are presented in Table 2. At day 12 approximately 66% inhibition of p24 antigen production was observed in 2.10.16R1 cells compared with U937 control cells. The cells were then maintained with 3- to 4-day Ficoll separation of the live from dead cells (as described above) until day 21. At this time, 2.10.16R1 showed no evidence of p24 antigen and the cells were 100% cell viable, as determined by trypan blue staining (data not shown). In contrast, 2.2.78 control cells (U937 control, Table 2) produced greater that 1,800 pg of p24 per ml, and parental U937 cells (not shown) weren't viable.

Third challenge of 2.10.16 cells. In this experiment, the pooled clone population identified as 2.10.16R1 from day 21 of the second challenge experiment (now referred to as clone 2.10.16R2) was infected with the IIIB strain of HIV-1 as described for the second challenge experiment. No p24 product was obtained following exposure of 2.10.16R2 cells to an MOI of 0.01, while high-level infection was seen in both parental U937 cells and control 2.2.78 cells.

In situ RT-PCR assay for U1/HIV antisense RNA in 2.10.16R2 (postchallenge) cells. In situ RT-PCR performed on the 2.10.16R2 cells showed that over 90% of these cells expressed U1/HIV antisense RNA at a detectable level (Fig. 2B), as determined by using HU1-1 (see Materials and Methods) as the upstream primer and either HVA-2 or HVC-2 as the downstream primer. Using HVB-2 or HVC-2 as the downstream primer gave similar results. The production of U1/HIV antisense in essentially all of the postchallenge cells is in contrast to a detection rate of 10 to 20% in prechallenge cells (Fig. 2D).

Tat-activated expression in cells transfected with U1/HIV antisense constructs. The effect of U1/HIV antisense constructs on Tat-activated expression was determined in transient expression assays measuring Tat-activated expression of CAT (see Materials and Methods). In unchallenged cells (Fig. 3A), whether clone 2.10.16, which carries the multitargeting construct pNDU1A,B,C), or any cells carrying the single targeting constructs (4.12, 5.34, or 8.54, carrying HIV antisense sequence A, B, or C, respectively), expression was inhibited. Postchallenge cells 2.10.16R2 (Fig. 3B) also demonstrated inhibition or Tat-activated CAT expression.

Assay for CD4$^+$ antigen of the surface of 2.10.16R2 cells. To determine whether the 2.10.16R2 cells had retained the CD4$^+$ antigen, these cells were examined in flow cytometry after treatment with mouse anti CD4$^+$ antibody (Leu-3a; Becton Dickinson) and fluoresceinated goat anti-mouse (Tago). The analysis showed that 45% of 2.10.16R2 cells contained CD4$^+$ antigen at a measurable level. This is equivalent to the 50 to 60% level normally observed in the parent U937 cells and indicates that the observed resistance to HIV-1 cannot be attributed to loss of the receptor protein CD4$^+$.

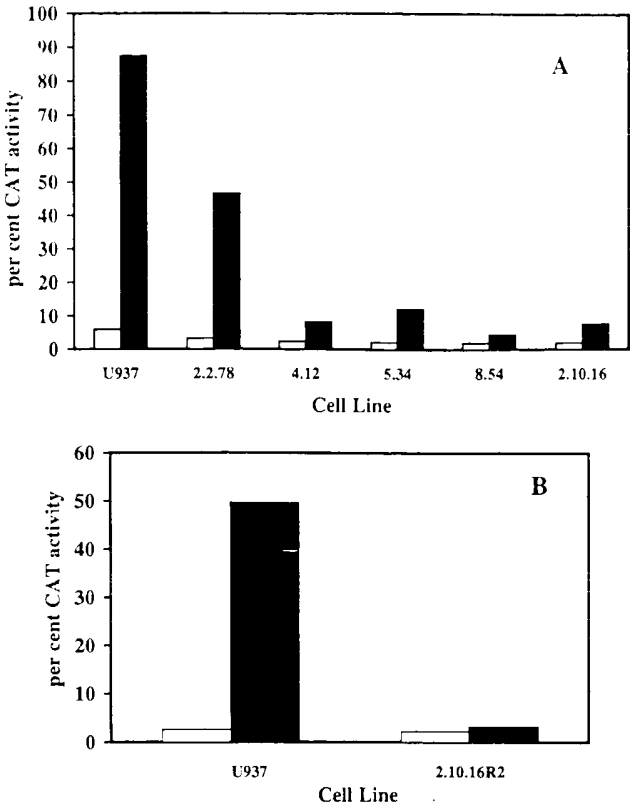

FIG. 3. Effect of U1/HIV antisense RNA on Tat-activated CAT expression. Transient expression assays for CAT were performed as described in Materials and Methods. Empty bars represent CAT expression in the absence of Tat; filled bars represent CAT expression in the presence of Tat. (A) CAT activity in prechallenge cells. The cell lines are described in the text. The 2.2.78 cells are transfected with the control plasmid pNDU1D. (B) CAT activity in postchallenge cells 2.10.16R2.

TABLE 2. Second HIV challenge of 2.10.16R1 and the parental U937 control cells

Cells	p24 (pg/ml)		
	Day 9	Day 12	Day 21
U937 control	3	5.1	>1,800
2.10.16R1	0	14.3	0

PCR assay for HIV-1 sequences in 2.10.16R2. Further evidence that the virus is not present in this cell population resulted from PCR assays for HIV nucleic acid. In assays using DNA PCR (Perkin-Elmer) and the manufacturer's primers which recognize the *gag* coding region of the BAL strain of HIV-1, no viral DNA was detected in 2.10.16R2 cells. As can be seen in Fig. 4A, by using various numbers of untreated U1.1A cells as a positive control, as few as 10^1 to 10^2 U1.1A cells can be detected. However, amplification products of DNA from 10^5 2.10.16R2 cells showed no HIV DNA. Similarly, PCR in situ hybridization using primers specific for the *gag* gene did not detect the provirus in any of the 2.10.16R2 cells. This is in contrast to a detection rate of over 90% in the HIV-infected pooled lymphocytes (data not shown).

PCR assay for presence of the U1/HIV antisense construct in 2.10.16R2 cells. 2.10.16R2 cells maintained for 60 days postchallenge were assayed for the presence of the U1/HIV antisense DNA by PCR assay. This assay used a primer pair representing U1 transcript region sequences which occur on either

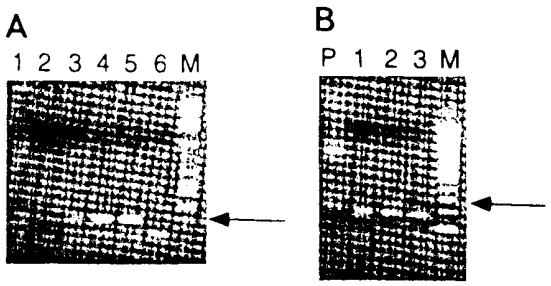

FIG. 4. (A) PCR assay for HIV-1 *gag* in HIV-infected control cells and in 2.10.16R1 cells and 2.10.16R2 cells. The arrow indicates the HIV-1 *gag* amplicon (147 bp). Lane 1, 10^2 U1.1A cells (promonocytic cells with two stably integrated copies of HIV); lane 2, 10^3 U1.1A cells; lane 3, 10^4 U1.1A cells; lane 4, 10^5 U1.1A cells; lane 5, 10^4 2.10.16R1 cells (from day 31 after initial HIV infection); lane 6, 10^5 2.10.16R2 cells (from day 60 after two challenges by HIV-1). (B) PCR assay for the pNDU1A,B,C sequences. The arrow indicates the amplicon for U1/HIV antisense sequences (199 to 206 bp). Lane P, amplification of a 1:1 mixture of DNA from pNDU1A,B,C and from pUC13 carrying the U1 operon; lane 1, amplification of DNA from 2.10.16R2 cells from 60 days (after two HIV-1 challenges); lane 2, amplification of DNA from 2, 2.10.16R1 cells from 31 days after the initial HIV-1 challenge; lane 3, amplification of DNA from U937 cells. Lanes M contain molecular weight markers.

side of the inserted antisense sequences. An amplicon derived from the U1 sequence (without an insert) would be 179 bp in length. An amplicon derived from U1 DNA with an inserted HIV antisense sequence would be 206 bp for sequence A, 200 bp for sequence B, and 199 bp for sequence C, or 20, 21, and 27 bp longer than the U1 amplicon, respectively. Figure 4B presents the results of amplification of 2.10.16R2 cells from day 60 postchallenge (lane 1) and 2.10.16R1 cells from day 31 postchallenge (lane 2). DNA derived from these cells yields two bands on electrophoresis (Fig. 4B). The upper band is approximately 200 bp and is absent in U937 cells (which contain no U1/HIV antisense sequences and only endogenous U1 gene sequences). The two bands are coincident with bands produced from an equal mixture of DNAs from pNDU1A,B,C and from pUC13 with the inserted U1 operon (lane P). These data are consistent with the presence of the U1/HIV antisense sequences in clones 2.10.16R1 and 2.10.16R2.

DISCUSSION

We report human HIV-1-resistant immune cells in culture that show a high level of resistance to multiple challenges by HIV for a prolonged period. During the challenge period, these cells maintained viability, showed no evidence of latent viral DNA, and retained their $CD4^+$ phenotype. These data demonstrate that HIV-1-resistant $CD4^+$ cells can be developed by using U1/HIV antisense constructs.

Attempts have been made by others to use genetic antisense constructs as a means of obtaining immune cells that are resistant to HIV-1 by utilizing antisense sequences against one specific HIV-1 target gene (2) or single RNA transcripts with antisense sequences against more than one HIV-1 target. In contrast to these studies, which showed a short period of HIV-1 resistance followed by a period of virus growth and ultimate cell destruction (6, 19), we have been successful in conferring a stable, HIV-1 resistance to immune cells in culture by combining independent multitargeting with the use of U1 snRNA as an antisense carrier.

The use of U1 snRNA provides a way to localize antisense transcripts in the cell nucleus as a means for delivery of anti-HIV antisense RNA to target sequences. While an active population of U1/antisense transcripts may be present in the cytoplasm at a concentration below the level of detection, the in situ analyses presented herein indicate that a vast majority of the U1/HIV antisense RNA is located in the nucleus. Nuclear location could be advantageous for effectiveness since higher concentrations of the antisense RNA can be maintained in the smaller volume of the nucleus, interactions with mRNA can occur prior to or during processing and translation, and there would be no possibility of competition with messenger-binding ribosomes. U1 snRNA also provides its inherent properties of stability and high level of transcription. Furthermore, several U1 antisense cassettes, each comprising approximately 750 bp, can be incorporated into a single vector.

To retain U1 snRNA's inherent properties for the delivery of antisense sequences, we attempted to maintain the original size as much as possible by using replacement of U1 sequences with antisense sequences. The resulting increases were only 20, 21, and 27 bases to the three U1/HIV antisense constructs. Although this substitution disrupts the formation of loops I and II, which are responsible for binding of U1-specific proteins, it allows exposure of the antisense sequences to potential target RNA molecules. Also, insertion near the 5′ end of the transcript reduces the likelihood of disrupting secondary structures that may be critical for binding proteins that are responsible for reimportation to the nucleus. In fact, computer predictions of secondary structures of the hybrid U1/HIV antisense RNA molecules show no perturbation of the Sm region or loops III and IV.

The U937 immune cells carrying U1/HIV antisense RNA (2.10.16) were obtained as a cell population enriched for resistance to G418. Since selection was made for G418, and not for U1/HIV antisense expression, this population of cells would be expected to contain cells that vary widely in antisense expression. This variation in expression among clones has been seen previously by Muller et al. (12) and by Szabo et al. (22), who introduced foreign genes into U937 cells as part of constructs expressing G418 resistance. Thus, it is not surprising that the first HIV-1 challenge resulted in virus growth in a portion of the heterogeneous 2.10.16 cell population. Evidence that this population is indeed heterogeneous is supported by in situ RT-PCR assays which show that approximately 10 to 20% of the cells produce detectable levels of each of the U1/HIV antisense RNAs. HIV-1 replication was significantly reduced in the second challenge, and during the third challenge no virus growth was detected as indicated by p24 assay.

The development of this resistance to HIV-1 correlated with the observation that essentially all 2.10.16R2 cells produced U1/HIV antisense RNA, as shown by in situ analysis. Further evidence that U1/HIV antisense RNA is responsible for this HIV-1 resistance was provided by transient expression assays for Tat-activated expression of CAT in 2.10.16R2 cells. Here, 2.10.16R2, compared with U937 cells and U937 cells carrying a control construct (2.2.78), markedly inhibited Tat activated expression.

Although the U1 antisense was designed to act in the nucleus, the inability to detect provirus sequences in postchallenge cells suggests the possibility that the U1/HIV antisense is capable of acting in the cytoplasm prior to HIV-1 integration. The presence of active antisense in the cytoplasm could result from the natural kinetics of U1 processing (23) wherein the U1/HIV antisense chimeric molecules are present in the cytoplasm prior to reimportation to the nucleus.

We have successfully achieved stable resistance to HIV in human immune cells in culture. Independent multitargeting was used to combat the variability and mutability of the virus. This approach also provided independent expression of each of the target sequences from an independent and specific pro-

moter, taking advantage of high rates of synthesis. The choice of U1 as an antisense carrier provided structural stability and nuclear localization. This successful approach in cell culture is being developed as means of achieving a high level of stable resistance in patient cells for the purpose of developing an ex vivo therapy for treating HIV infections.

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